In Vitro Acquisition of Secondary Azole Resistance in *Aspergillus fumigatus* Isolates after Prolonged Exposure to Itraconazole: Presence of Heteroresistant Populations

Pilar Escribano,^{a,b,c} Sandra Recio,^{a,b} Teresa Peláez,^{a,b,c,d} Milagros González-Rivera,^{b,e} Emilio Bouza,^{a,b,c,d} and Jesús Guinea^{a,b,c,d}

Clinical Microbiology and Infectious Diseases Department, Hospital General Universitario Gregorio Marañón, Universidad Complutense de Madrid, Madrid, Spain^a; Instituto de Investigación Sanitaria del Hospital Gregorio Marañón, Madrid, Spain^b; CIBER de Enfermedades Respiratorias, Palma de Mallorca, Spain^c; Microbiology Department, School of Medicine, Universidad Complutense de Madrid, Madrid, Spain^d; and Sequencing Unit, Hospital General Universitario Gregorio Marañón, Universidad Complutense de Madrid, Madrid, Spain^e

Secondary resistance to azoles in *Aspergillus fumigatus* isolates from patients taking long-term itraconazole therapy has been described. We studied the acquisition of secondary azole resistance in 20 *A. fumigatus* isolates with no mutations at codon 54, 98, 138, 220, 432, or 448 in the *cyp51A* gene. Adjusted conidium inocula (3×10^7 CFU/ml) of each isolate were prepared and progressively or directly exposed to increasing itraconazole concentrations, ranging from 0.5 μ g/ml to 16 μ g/ml. Itraconazole, voriconazole, and posaconazole MICs were determined using the CLSI M38-A2 procedure before (MIC_{initial}) and after (MIC_{final}) exposure to itraconazole. In both procedures, the MIC_{final} was significantly higher than the MIC_{initial}. However, after progressive exposure to itraconazole, the MICs of the three azoles were higher than after direct exposure. No mutations were found at codon 54, 98, 138, 220, 432, or 448 in the *cyp51A* gene of isolates growing at the highest concentration of itraconazole. More concentrated conidium inocula (2×10^9 CFU/ml) plated in itraconazole at 4 μ g/ml revealed the presence of heteroresistant populations in two initially wild-type isolates. These isolates became resistant to itraconazole and posaconazole only after use of the concentrated inoculum. These heteroresistant isolates harbored a mutation at codon G54, and the MICs of itraconazole and posaconazole were >16 μ g/ml. In all procedures, *A. fumigatus* short tandem repeat (STRAf) typing was used to demonstrate that the genotype did not change before or after exposure to itraconazole.

Recent reports have indicated increased isolation of clinical and environmental *Aspergillus fumigatus* isolates showing phenotypic resistance to one or more azoles (13, 23, 25, 29). Azole-resistant *A. fumigatus* isolates commonly show mutations in the *cyp51A* gene at codons G54, L98, G138, M220, G432, and G448 (1, 3, 10, 15–17). The presence of mutations at other codons does not seem to be related to fungal resistance to azoles (24).

In the clinical setting, the development of secondary itraconazole resistance in *A. fumigatus* isolates has been documented mostly in patients with chronic lung diseases harboring high-conidium inocula (e.g., aspergilloma) and who were receiving long-term therapy with itraconazole (2, 3, 4, 13–15). Most of the isolates showing phenotypic resistance to itraconazole had mutations in the *cyp51A* gene.

In a recent report on *A. fumigatus* isolates that were susceptible or intermediate to itraconazole, we found mutations in *cyp51A* at positions other than G54, L98, G138, M220, G432, and G448. We hypothesized that these mutations could play a role in the prevention of the acquisition of new mutations in *cyp51A*, especially at positions conferring resistance to one or more azoles (11). We studied potential acquisition of *in vitro* secondary azole resistance in *A. fumigatus*, the role of mutations in *cyp51A* (at positions other than G54, L98, G138, M220, G432, and G448) in the development of secondary resistance, and the effect of inoculum size on detection of heteroresistant populations.

(This study was partially presented at the 21st European Congress of Clinical Microbiology and Infectious Diseases [ECCMID]-27th International Congress of Chemotherapy [ICC] [oral session O479], 2011, Milan, Italy.)

MATERIALS AND METHODS

Isolates studied. We studied 20 *A. fumigatus* isolates from the air ($n = 6$) or from clinical samples. Strains were identified by sequencing the β -tubulin gene; the *cyp51A* gene sequence was obtained as previously described (11). Fourteen clinical isolates were isolated from 13 patients, 6 of whom had been diagnosed with proven or probable invasive aspergillosis. The isolates had a wild-type *cyp51A* gene sequence ($n = 10$, referred to as WT-1 to WT-10) or had one or more nucleotide substitutions (referred to with the suffix MUT) (Table 1). For each mutant strain, we randomly selected a wild-type strain with the same itraconazole MIC.

Antifungal susceptibility testing. Pure itraconazole (Janssen Pharmaceutical Research and Development, Madrid, Spain), posaconazole (Merck Research Laboratories, Rahway, NJ), and voriconazole (Pfizer Pharmaceutical Group, New York, NY) were used. For each drug-strain combination, the MIC was obtained in 3 to 6 independent experiments according to the CLSI M38-A2 document (5). The modal MIC for each drug-strain combination was chosen (MIC_{initial}).

As clinical breakpoints for triazoles and *Aspergillus* are not yet available for the CLSI procedure, isolates were classified according to breakpoints recently proposed by Verweij et al. using EUCAST methodology (28). For itraconazole and voriconazole, breakpoints were as follows: <2 μ g/ml, susceptible; 2 μ g/ml, intermediate; and >2 μ g/ml, resistant. For

Received 3 March 2011 Returned for modification 23 September 2011

Accepted 9 October 2011

Published ahead of print 17 October 2011

Address correspondence to Jesús Guinea, jguineaortega@yahoo.es.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.00301-11

TABLE 1 Mutations found in the *cyp51A* genes of the 10 *A. fumigatus* isolates prior to exposure to itraconazole

Strain ^a	Cyp51A amino acid substitution(s)	Source (clinical significance)
1-MUT	D245D	Air
2-MUT	N479D	Air
3-MUT	F165L	Air
4-MUT	N248K	Air
5-MUT	D262Y	Clinical (nonsignificant)
6.1-MUT	M172V, G89G, L358L, C454C, G497G	Clinical (proven invasive aspergillosis)
6.2-MUT	F46Y, M172V, E427K, G89G, L358L, C454C, G497G	Clinical (proven invasive aspergillosis)
7-MUT	F46Y, M172V, E427K, G89G, L358L, C454C, G497G	Clinical (nonsignificant)
8-MUT	F46Y, M172V, N248T, D255E, E427K, G89G, L358L, C454C	Clinical (probable invasive aspergillosis)
9-MUT	F46Y, M172V, N248T, D255E, E427K, G89G, L358L, C454C	Clinical (probable invasive aspergillosis)

^a Isolates 6.1 and 6.2 were from the same patient.

posaconazole, breakpoints were as follows: <0.5 $\mu\text{g/ml}$, susceptible; 0.5 $\mu\text{g/ml}$, intermediate; and >0.5 $\mu\text{g/ml}$, resistant.

Acquisition of secondary azole resistance. Each isolate was grown on an azole-free Sabouraud dextrose agar plate after sporulation. Conidium suspensions were prepared in sterile distilled water by stirring with a wetted cotton swab. The densities of the inocula were spectrophotometrically adjusted to an optical density of 0.09 to 0.11. Each adjusted inoculum was counted using a Neubauer chamber; the mean number of conidia per ml was 3×10^7 . Twenty microliters of the inoculum was subcultured on plates containing itraconazole at concentrations ranging from 0.5 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$ in two different experiments.

(i) **Progressive exposure to itraconazole (experiment A).** The inoculum was transferred to a plate containing itraconazole at a concentration one 2-fold dilution below the $\text{MIC}_{\text{initial}}$. The plates were incubated at 35°C for 1 week, and if fungal growth with sporulation was observed, an adjusted suspension was further prepared directly from the plate. The new suspension was propagated to another plate containing the next 2-fold itraconazole concentration and incubated again at 35°C for 1 week. This procedure was serially repeated until fungal growth was inhibited or until propagation to the plate containing 16 $\mu\text{g/ml}$ of itraconazole (Fig. 1).

(ii) **Direct exposure to itraconazole (experiment B).** The inoculum was directly transferred to plates containing itraconazole at a concentration of 0.5 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 2 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$, 8 $\mu\text{g/ml}$, or 16 $\mu\text{g/ml}$ (Fig. 1). The plates were also incubated at 35°C for 1 week.

To investigate whether the size of the conidium inoculum could have an effect on the acquisition of secondary resistance to azoles, we prepared a more concentrated inoculum (2×10^9 CFU/ml) for each isolate, which was streaked directly onto a plate containing itraconazole at a concentration of 4 $\mu\text{g/ml}$. Other authors have previously used agar plates containing itraconazole at this concentration to screen for the presence of azole-

resistant *A. fumigatus* isolates (23, 25). The plates were incubated at 35°C for 7 days.

To investigate whether the susceptibility of the isolates changed after progressive or direct exposure to itraconazole, we prepared conidium inocula directly from the itraconazole-containing plates at the highest concentration allowing fungal growth. The MICs of the three azoles ($\text{MIC}_{\text{final}}$) were obtained according to the CLSI M38-A2 procedure (5). We used the same conidium suspension as for the $\text{MIC}_{\text{final}}$ determination to obtain the *cyp51A* gene sequence and *A. fumigatus* short tandem repeat (STRAf) type of each isolate (4, 9). The nine STRAf markers were used to detect azole-resistant isolates that could contaminate the susceptible isolates. $\text{MIC}_{\text{initial}}$ and $\text{MIC}_{\text{final}}$ were compared by means of the Wilcoxon signed rank test using the PASW Statistics 18.0 software package (SPSS Inc., Chicago, IL). The comparisons were considered statistically significant at a P value <0.05 .

RESULTS

After progressive exposure (experiment A) and direct exposure (experiment B), most of the isolates (19/20) were able to grow on itraconazole-containing agar plates. After progressive exposure, the proportions of isolates able to grow on itraconazole-containing plates at concentrations of 2 $\mu\text{g/ml}$, 4 $\mu\text{g/ml}$, 8 $\mu\text{g/ml}$, and 16 $\mu\text{g/ml}$ were 95%, 75%, 70%, and 50%, respectively. However, after direct exposure, these values were 95%, 50%, 20%, and 10%, respectively.

The $\text{MIC}_{\text{initial}}$ s and $\text{MIC}_{\text{final}}$ s of itraconazole, voriconazole, and posaconazole for the 20 isolates are shown in Table 2. After progressive and direct exposure, we observed a statistically significant increase in the geometric mean MICs of the three azoles (Table 2).

FIG 1 Representation of progressive exposure (experiment A) or direct exposure (experiment B) to itraconazole (ITC) to induce secondary azole resistance in 20 *Aspergillus fumigatus* isolates. In both experiments, 20 μl of the adjusted conidium suspension of 3×10^7 CFU/ml was used. All plates were incubated at 35°C for 1 week.

TABLE 2 Susceptibilities of the 20 *A. fumigatus* strains to itraconazole, voriconazole, and posaconazole^a

Parameter	Itraconazole				Voriconazole				Posaconazole			
	Range	Geometric mean	90%	P ^b	Range	Geometric mean	90%	P ^b	Range	Geometric mean	90%	P ^b
MIC _{initial} (μg/ml)	1–2	1.40	2		0.25–2	1.01	2		0.5–1	0.63	1	
MIC _{final} (μg/ml) after:												
Progressive exposure	1–>16	15.90	>16	<0.002	1–8	3.42	8	<0.002	0.5–2	1.29	2	<0.001
Direct exposure	2–>16	5.16	8		1–4	1.79	4		1–2	1.11	2	

^a Susceptibility (geometric mean MIC) before and after progressive exposure (experiment A) or direct exposure (experiment B) to itraconazole in agar plates was obtained determined by means of the CLSI M38-A2 procedure (5).
^b P value for comparison between the geometric mean MIC before and after exposure to itraconazole.

However, progressive exposure to itraconazole led to a higher geometric mean MIC than direct exposure for all three azoles (itraconazole, 15.9 versus 5.16 μg/ml [*P* = 0.021]; voriconazole, 3.42 versus 1.79 μg/ml [*P* = 0.004]; and posaconazole, 1.29 versus 1.11 μg/ml [*P* = 0.317]). The geometric mean MIC_{final} of itraconazole was more affected than the MIC_{final} of voriconazole or posaconazole after fungal growth in the itraconazole-containing plates. We did not observe differences in the ability to grow in plates containing itraconazole at high concentrations or in the MIC_{final} between isolates harboring or not harboring mutations in the *cyp51A* gene.

According to the breakpoints recently proposed by Verweij et al. (28), the isolates were classified as itraconazole susceptible (60%) or intermediate (40%) before exposure. After both progressive and direct exposures, we observed an increase in the proportion of resistant isolates for all three azoles. The percentages of isolates resistant to itraconazole and voriconazole were higher after progressive exposure (79% and 52.6%, respectively) than after direct exposure (52.6% and 10.5%, respectively). Posaconazole showed approximately the same percentage of resistance after both exposures (28) (Table 3).

Although most of the isolates showed higher MICs of itraconazole, voriconazole, and posaconazole, no additional mutations were found in the sequence of the *cyp51A* gene. STRAf typing revealed no contaminations with azole-resistant isolates occurring during exposure to itraconazole. STRAf typing performed in intermediate steps of progressive exposure to itraconazole also showed no contaminations (data not shown).

Additionally, concentrated inocula of each isolate were prepared and streaked onto agar plates containing itraconazole at a

concentration of 4 μg/ml. After 1 week of incubation, all isolates were able to grow on the plates. The antifungal susceptibilities of these isolates according to CLSI M38-A2 (5), in terms of MIC_{final} range, geometric mean MIC_{final}, and MIC_{final90}, respectively, were as follows: for itraconazole, 1 to >16 μg/ml, 8.85 μg/ml, and >16 μg/ml; for voriconazole, 0.25 to 4 μg/ml, 1.46 μg/ml, and 2 μg/ml; and for posaconazole, (0.5 to >16 μg/ml, 4.1 μg/ml, and 2 μg/ml. For all the strains, differences between the geometric mean MICs before and after exposure to itraconazole reached statistical significance only for itraconazole and posaconazole and not for voriconazole. Surprisingly, in two isolates, the MIC_{final} for itraconazole and posaconazole was >16 μg/ml, and the MIC_{final} for voriconazole was one 2-fold dilution lower than the MIC_{initial} (Table 4). After sequencing of the *cyp51A* genes of these two initially wild-type isolates, two different point mutations were found at position G54 (G54R and G54W). The STRAf typing assay ensured that both resistant isolates were identical to their parental strains. The isolates were from two different patients. In one, the isolation of *A. fumigatus* (WT-10) was considered nonsignificant, and the patient did not receive antifungal agents. The other patient, who had chronic obstructive pulmonary disease and was diagnosed with probable invasive aspergillosis, received antifungal treatment with voriconazole after isolation of *A. fumigatus* (WT-2) and survived. The sequence analysis of the *cyp51A* gene showed no changes in the remaining isolates compared to the sequences of the parental isolates.

TABLE 3 Percentages of isolates classified as susceptible, intermediate, or resistant to itraconazole, voriconazole, and posaconazole

Parameter	% of isolates ^a								
	Itraconazole:			Voriconazole:			Posaconazole:		
	S	I	R	S	I	R	S	I	R
MIC _{initial}	60	40	0	85	15	0	0	75	25
MIC _{final} ^b									
Progressive exposure	10.5	10.5	79	5.2	42.2	52.6	0	5.3	94.7
Direct exposure	0	47.4	52.6	42.1	47.4	10.5	0	0	100

^a Breakpoints were those suggested by Verweij et al. (28). Breakpoints for itraconazole and voriconazole were as follows: <2 μg/ml, susceptible (S); 2 μg/ml, intermediate (I); and >2 μg/ml, resistant (R). Breakpoints for posaconazole were as follow: <0.5 μg/ml, susceptible; 0.5 μg/ml, intermediate; >0.5 μg/ml, resistant).
^b One isolate was not able to grow on itraconazole-containing agar plates.

TABLE 4 Susceptibilities to itraconazole, voriconazole, and posaconazole of the two *A. fumigatus* strains in which a mutation was found at codon G54

Strain and antifungal ^a	MIC _{initial} (μg/ml)	MIC _{final} (μg/ml)		
		Progressive exposure	Direct exposure	Direct concentrated exposure
2-WT (G54R)				
Itraconazole	1	>16	2	>16
Voriconazole	0.5	4	1	0.25
Posaconazole	0.5	1	1	>16
10-WT (G54W)				
Itraconazole	2	2	2	>16
Voriconazole	1	2	2	0.5
Posaconazole	0.5	1	2	>16

^a The mutation was found only after growing a concentrated conidium suspension of the isolates on plates containing itraconazole at a concentration of 4 μg/ml.

The genetic stability of the G54 mutation and the phenotypic resistance to itraconazole and posaconazole in these two isolates were further studied. We transferred the isolates to Sabouraud dextrose agar plates without itraconazole weekly for 5 weeks. The plates were incubated at 35°C. After five transfers, mutant isolates showed the same itraconazole and posaconazole MICs ($>16 \mu\text{g/ml}$), kept the mutation at codon G54, and were observed to belong to the same STRAf type as the progenitor. We also studied the stability of the itraconazole resistance ($>16 \mu\text{g/ml}$) in two isolates (WT-3, and 6.1-MUT) that did not harbor the mutation at codon G54. After 5 weeks of propagation to Sabouraud dextrose agar plates without itraconazole, the isolates showed MICs similar to the MIC_{initial}s ($1 \mu\text{g/ml}$ for WT-3 and $2 \mu\text{g/ml}$ for 6.1-MUT).

DISCUSSION

Azoles are widely used for the prevention and treatment of invasive aspergillosis, and the decision to administer one drug or another has not been influenced by *A. fumigatus* resistance to this family. However, recent studies reported infections caused by azole-resistant *A. fumigatus* isolates (25, 26). In fact, azole resistance in *A. fumigatus* is complicating the management of patients with invasive aspergillosis, and further data are necessary to increase our understanding of this problem.

Azole-resistant isolates usually show specific mutations at codons G54, L98, G138, M220, G432, and G448 (1, 3, 10, 15–17). The presence of a substitution at amino acid L98, together with a 34-bp tandem repeat in the gene promoter, is commonly found in resistant isolates from the Netherlands (23, 29). This mutation has been found in clinical and environmental isolates, even in samples from patients who had never received azoles. These observations suggest that this primary resistant genotype could have been generated in the environment and is now spreading over wider areas (12, 19, 30). In contrast, other authors report the presence of itraconazole-resistant isolates in patients receiving long-term azole therapy in whom the initial isolates were susceptible (7, 13, 15). In some of these patients, a preexisting cavity colonized by *A. fumigatus* was observed. The presence of an anatomical site at which conidiogenesis is possible, together with prolonged exposure to itraconazole, creates a setting in which secondary azole resistance can develop.

In a previous study, we examined the role of the mutations found in the *cyp51A* gene at positions other than G54, L98, G138, M220, G432, and G448 (Table 1) (11). We found that these mutations were not involved in antifungal resistance. However, we hypothesized that after exposure to subinhibitory and increasing itraconazole concentrations, these mutations could prevent isolates from acquiring new mutations at positions G54, L98, G138, M220, G432, and G448. Our isolates were progressively or directly exposed to increasing itraconazole concentrations, ranging from $0.5 \mu\text{g/ml}$ to $16 \mu\text{g/ml}$. The concentration of $0.5 \mu\text{g/ml}$ is commonly found in patients who receive oral itraconazole (22). A concentration of $5.5 \mu\text{g/ml}$ has been measured in alveolar cells, and concentrations of greater than $10 \mu\text{g/ml}$ have also been measured in serum samples (6, 21).

We observed that the MIC_{final} was significantly higher than the MIC_{initial}. This effect was independent of the presence of the *cyp51A* mutations in our isolates (Table 1). However, itraconazole was the most affected agent after exposure, suggesting that isolates can adapt to the presence of a high itraconazole concentration without this being reflected in sequence changes in *cyp51A*. In-

deed, isolates showing an itraconazole MIC of $>16 \mu\text{g/ml}$ with no mutation at codon G54 experienced a decrease in the itraconazole MIC after propagation without itraconazole. Although we did not study the mechanism of itraconazole resistance operating in these two isolates, we cannot exclude the presence of overexpression of the *cyp51A* gene or overexpression of efflux pumps, as previously described (2, 8, 20).

When more concentrated inocula were studied, we found that the itraconazole-containing plates ($4 \mu\text{g/ml}$) were able to select two isolates with mutations at codon G54. These mutations led to a phenotype that was consistent with resistance to itraconazole and posaconazole (MIC of $>16 \mu\text{g/ml}$) and susceptibility to voriconazole. The isolates were of the same STRAf type before and after exposure. The resistance conferred by the mutation at position G54 was stable. In order to study the reproducibility of this finding, we repeated the procedure three times for the two isolates, and the mutations were always found.

The selection of heteroresistant populations was evidenced only after the propagation of concentrated conidium inocula on plates containing itraconazole at a concentration of $4 \mu\text{g/ml}$. These findings suggest that a low proportion of the whole conidium population in these two isolates was resistant to itraconazole and posaconazole. Therefore, the presence of these heteroresistant populations can be evidenced only when high numbers of conidia are present. Others have previously used this procedure to screen for the presence of azole-resistant *A. fumigatus* isolates (23). In fact, propagation of concentrated *A. fumigatus* conidium suspensions on plates containing itraconazole at a concentration of $4 \mu\text{g/ml}$ can reveal the presence of heteroresistant populations. However, the CLSI M38-A2 method (5) is probably not sufficiently sensitive to uncover the presence of heteroresistant subpopulations due to the adjusted inoculum used.

Heteroresistance has been well documented in bacteria such as *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Streptococcus pneumoniae* (18, 31, 32). The same phenomenon has recently been described in *Cryptococcus gatti* (27). However, to our knowledge, this is the first report of *in vitro* heteroresistance to azoles in *A. fumigatus*. In light of these findings, we cannot exclude the possibility of selection of a heteroresistant population in patients on long-term azole therapy and with specific clinical manifestations, such as aspergilloma, in which large amounts of *Aspergillus* can be found (3, 4, 14). During antifungal treatment, the minority resistant population may be enriched, and this could explain the appearance of mutations in a very conservative region of the *cyp51A* gene occurring in a single genotype during antifungal treatment (3, 4).

Although the number of isolates studied was low, we found heteroresistant populations only in isolates with no previous mutations in the *cyp51A* gene, suggesting that the presence of mutations not conferring resistance to azoles can protect the isolates from accumulating additional mutations at positions leading to azole resistance. This finding should be confirmed in future studies including large numbers of isolates.

Our study has several limitations. We did not study the potential acquisition of secondary resistance to azoles in very azole-susceptible isolates (with itraconazole MICs of $<1 \mu\text{g/ml}$). We did not study mechanisms of resistance to azoles other than the presence of mutations in *cyp51A*. However, one of the strengths of the study was the use of a very discriminatory and reproducible genotyping assay (STRAf) to ensure that the isolates with acquired

secondary azole resistance, and the heteroresistant populations, were the same as the progenitor isolates.

In conclusion, we highlight two important observations in patients receiving long-term itraconazole, namely, the acquisition of secondary azole resistance in *A. fumigatus* isolates and the selection of a minority resistant population harboring mutations at codon G54 of the *cyp51A* gene.

ACKNOWLEDGMENTS

We thank Thomas O'Boyle for editing and proofreading the article. We are grateful to Ainhoa Simón Zárate for her participation in the sequencing analysis.

This study does not present any conflicts of interest for its authors.

This study was partially financed by grants from the Fondo de Investigación Sanitaria (FIS) PI070198 (Instituto de Salud Carlos III). Jesús Guinea (CP09/00055) and Pilar Escribano (CD09/00230) are contracted by the FIS. Ainhoa Simón Zárate holds a grant from the Fondo de Investigaciones Sanitarias (Línea Instrumental Secuenciación). The 3130xl Genetic Analyzer was partially financed by grants from Fondo de Investigaciones Sanitarias (IF01-3624 and IF08-36173).

REFERENCES

- Alanio A, et al. 2011. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. *J. Antimicrob. Chemother.* 66:371–374.
- Arendrup MC, et al. 2010. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS One* 5:e10080.
- Bellete B, et al. 2010. Acquired resistance to voriconazole and itraconazole in a patient with pulmonary aspergilloma. *Med. Mycol.* 48:197–200.
- Chen J, Li H, Li R, Bu D, Wan Z. 2005. Mutations in the *cyp51A* gene and susceptibility to itraconazole in *Aspergillus fumigatus* serially isolated from a patient with lung aspergilloma. *J. Antimicrob. Chemother.* 55:31–37.
- Clinical Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard CLSI document M38-A2. Clinical and Laboratory Standards Institute, Wayne, PA.
- Conte JE, Jr, Golden JA, Kipps J, McIver M, Zurlinden E. 2004. Intrapulmonary pharmacokinetics and pharmacodynamics of itraconazole and 14-hydroxyitraconazole at steady state. *Antimicrob. Agents Chemother.* 48:3823–3827.
- Dannaoui E, et al. 2001. Acquired itraconazole resistance in *Aspergillus fumigatus*. *J. Antimicrob. Chemother.* 47:333–340.
- da Silva Ferreira ME, et al. 2004. In vitro evolution of itraconazole resistance in *Aspergillus fumigatus* involves multiple mechanisms of resistance. *Antimicrob. Agents Chemother.* 48:4405–4413.
- de Valk HA, et al. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. *J. Clin. Microbiol.* 43:4112–4120.
- Díaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodríguez-Tudela JL. 2003. A point mutation in the 14 α -sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* 47:1120–1124.
- Escribano P, Recio S, Peláez T, Bouza E, Guinea J. 2011. *Aspergillus fumigatus* strains with mutations in the *cyp51A* gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. *Antimicrob. Agents Chemother.* 55:2460–2462.
- Howard SJ, Arendrup MC. 2011. Acquired antifungal drug resistance in *Aspergillus fumigatus*: epidemiology and detection. *Med. Mycol.* 49(Suppl. 1):S90–S95.
- Howard SJ, et al. 2009. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. *Emerg. Infect. Dis.* 15:1068–1076.
- Howard SJ, Pasqualotto AC, Denning DW. 2010. Azole resistance in allergic bronchopulmonary aspergillosis and *Aspergillus* bronchitis. *Clin. Microbiol. Infect.* 16:683–688.
- Howard SJ, et al. 2006. Multi-azole resistance in *Aspergillus fumigatus*. *Int. J. Antimicrob. Agents* 28:450–453.
- Mellado E, García-Effron G, Alcázar-Fuoli L, Cuenca-Estrella M, Rodríguez-Tudela JL. 2004. Substitutions at methionine 220 in the 14 α -sterol demethylase (*Cyp51A*) of *Aspergillus fumigatus* are responsible for resistance in vitro to azole antifungal drugs. *Antimicrob. Agents Chemother.* 48:2747–2750.
- Mellado E, et al. 2007. A new *Aspergillus fumigatus* resistance mechanism conferring in vitro cross-resistance to azole antifungals involves a combination of *cyp51A* alterations. *Antimicrob. Agents Chemother.* 51:1897–1904.
- Morand B, Muhlemann K. 2007. Heteroresistance to penicillin in *Streptococcus pneumoniae*. *Proc. Natl. Acad. Sci. U. S. A.* 104:14098–14103.
- Mortensen KL, et al. 2010. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob. Agents Chemother.* 54:4545–4549.
- Nascimento AM, et al. 2003. Multiple resistance mechanisms among *Aspergillus fumigatus* mutants with high-level resistance to itraconazole. *Antimicrob. Agents Chemother.* 47:1719–1726.
- Patterson TF, et al. 1996. Systemic availability of itraconazole in lung transplantation. *Antimicrob. Agents Chemother.* 40:2217–2220.
- Smith J, Andes D. 2008. Therapeutic drug monitoring of antifungals: pharmacokinetic and pharmacodynamic considerations. *Ther. Drug Monit.* 30:167–172.
- Snelders E, et al. 2009. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl. Environ. Microbiol.* 75:4053–4057.
- Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJ. 2010. Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. *Antimicrob. Agents Chemother.* 54:2425–2430.
- Snelders E, et al. 2008. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med.* 5:e219.
- van der Linden JW, et al. 2009. Azole-resistant central nervous system aspergillosis. *Clin. Infect. Dis.* 48:1111–1113.
- Varma A, Kwon-Chung KJ. 2010. Heteroresistance of *Cryptococcus gattii* to fluconazole. *Antimicrob. Agents Chemother.* 54:2303–2311.
- Verweij PE, Howard SJ, Melchers WJ, Denning DW. 2009. Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. *Drug Resist. Updat.* 12:141–147.
- Verweij PE, Mellado E, Melchers WJ. 2007. Multiple-triazole-resistant aspergillosis. *N. Engl. J. Med.* 356:1481–1483.
- Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. 2009. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? *Lancet Infect. Dis.* 9:789–795.
- Wootton M, et al. 2001. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. *J. Antimicrob. Chemother.* 47:399–403.
- Yau W, et al. 2009. Colistin hetero-resistance in multidrug-resistant *Acinetobacter baumannii* clinical isolates from the Western Pacific region in the SENTRY antimicrobial surveillance programme. *J. Infect.* 58:138–144.